Short Communication

Endogenous Inhibitors for Pollen Germination and Pollen Tube Elongation in *Pinus densiflora* SIEB. et ZUCC.Satoshi KOBAYASHI, Tetsuo OZAWA
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It has been known that pollen of some species contains substances which inhibit the germination and pollen tube elongation.^{1,2)} Pollen grains of *Pinus densiflora* contained at least two kinds of growth inhibitors, and they suppressed the growth of the pollen tubes of this plant and some angiosperms.^{3,4)} The pollen of *Camellia sasanqua* and *Tulipa gesneriana* also contained inhibiting substances which affected pollen growth of the same as well as other species.⁵⁾ However, it remains obscure what substances are responsible for the regulation of pollen growth. We now report the isolation of a growth inhibitor from the pollen of *Pinus densiflora* and its estimation of biological activities.

Throughout the isolation procedure, the inhibitors were monitored by the modified hanging drop technique as follows.⁶⁾ Pollen grains of *Camellia japonica* were sown on a drop (10 μ l) of the growth medium containing pollen extracts to be tested on a microscope slide in a Petri dish with a moist filter paper. The growth medium was a 10% sucrose solution containing 10 ppm H_3BO_3 . After incubation for 90 min at 25°C, inhibitory activity was determined.

Pollen grains of *P. densiflora* (4.55 kg) collected around the University of Tsukuba, May 1980, were extracted with 27.6 liters of 80% methanol for several days at room temperature. The filtered extract was concentrated at

40°C under a reduced pressure to a small volume of aqueous solution and the concentrate was centrifuged at 28,000 $\times g$ for 15 min. The supernatant was shaken ten times with *n*-hexane. Only the hexane insoluble fraction showed inhibitory activity to the assay. Then the aqueous layer was fractionated with ethyl acetate as an usual way. The marked inhibitory activity was found in the acidic fraction. A portion (1.83 g) of the acidic fraction was subjected to silicic acid-celite 545 (1:1) adsorption chromatography using the solvent system benzene-ethyl acetate followed by ethyl acetate-methanol, increasing stepwise the ratio of the latter solvent in the both solvent systems. Two fractions from the eluates showed strong inhibitory activities for the pollen germination of *C. japonica*.

From the eluates with benzene-ethyl acetate (90:10), 3.2 mg of a crystalline compound (mp 118°C) with the inhibitory activity was isolated by concentration of the eluates followed by recrystallization from hexane and water. Its spectral data were as follows. High MS: Found; 122.0381 (M^+), Calcd. for $C_7H_6O_2$; 122.0368; UV λ_{max}^{MeOH} nm (ϵ): 226 (11,400), 266 (776), 272 (897), 279 (721); IR ν_{max}^{KBr} cm^{-1} : 3200~2300 (OH), 1910, 1785, 1675 (C=O), 1600 (C=C), 1580 (C=C), 1415, 1320, 1280, 1175, 1120, 1065, 1020, 925, 800, 695; NMR $\delta_{Me_4Si}^{CCl_4}$: 7.5 (3H, m, aromatic protons), 8.1 (2H, dd, $J=8$ and 2 Hz, aromatic protons); MS m/z : 122 (M^+), 105 ($M^+ - OH$), 78, 77 ($M^+ - COOH$), 74, 51, 50, 39. This compound was identified as benzoic acid from the spectral analyses and direct comparison the spectra with those of an authentic specimen.

On the other hand, on standing the concentrate of the eluate with benzene-ethyl acetate (50:50), crude crystals with inhibitory activity were obtained, and further purification of their is now in progress.

The effect of benzoic acid on the germination and pollen tube elongation in *C. japonica*, *C. sinensis*, *Impatiens balsamina*, *Lilium auratum* and *P. densiflora* were tested. The assay was conducted according to the method of Iwanami and Iwadare.⁷⁾ To deter-

TABLE I. EFFECTS OF BENZOIC ACID ON
GERMINATION AND TUBE ELONGATION
OF FIVE KINDS OF CULTURED
POLLEN

Pollen grains were obtained from freshly opened flowers of *Camellia japonica* (March 1980), *Pinus densiflora* (May 1980), *Lilium auratum* (July 1980), *Impatiens balsamina* (September 1980) and *Camellia sinensis* (October 1980), stored in a desiccator over silica gel at -17°C and used for bioassay. Experiments were repeated more than three times and mean values with standard error were presented.

Species	Concentration (ppm)	Germination (%)	Pollen tube length (mm)
<i>C. japonica</i>	0	90.8 $\pm$ 1.3	3.3 $\pm$ 0.2
	5	90.6 $\pm$ 0.4	2.7 $\pm$ 0.2
	10	90.2 $\pm$ 1.0	2.2 $\pm$ 0.1
	25	87.8 $\pm$ 1.4	2.3 $\pm$ 0.2
	50	87.0 $\pm$ 1.5	1.1 $\pm$ 0.3
	100	0.9 $\pm$ 0.3	0.1 $\pm$ 0.0
<i>C. sinensis</i>	0	68.8 $\pm$ 2.4	4.8 $\pm$ 0.1
	5	60.2 $\pm$ 2.6	4.7 $\pm$ 0.1
	10	57.3 $\pm$ 3.3	4.2 $\pm$ 0.1
	25	48.6 $\pm$ 4.0	3.3 $\pm$ 0.2
	50	43.0 $\pm$ 2.2	0.5 $\pm$ 0.1
	100	0	0
<i>I. balsamina</i>	0	93.3 $\pm$ 0.3	1.8 $\pm$ 0.1
	5	89.2 $\pm$ 1.2	1.7 $\pm$ 0.2
	10	83.1 $\pm$ 5.3	1.7 $\pm$ 0.2
	25	80.8 $\pm$ 4.8	1.1 $\pm$ 0.2
	50	64.0 $\pm$ 12.8	0.1 $\pm$ 0.1
	100	4.3 $\pm$ 0.8	0
<i>L. auratum</i>	0	66.6 $\pm$ 4.7	10.6 $\pm$ 0.1
	5	53.4 $\pm$ 12.5	10.0 $\pm$ 0.2
	10	45.5 $\pm$ 13.3	9.1 $\pm$ 0.9
	25	28.4 $\pm$ 12.8	5.8 $\pm$ 1.0
	50	21.1 $\pm$ 3.3	4.5 $\pm$ 0.2
	100	1.9 $\pm$ 0.6	0
<i>P. densiflora</i> ^a	0	57.2 $\pm$ 2.4	143.8 $\pm$ 4.0
	5	39.0 $\pm$ 2.0	89.5 $\pm$ 4.5
	10	41.7 $\pm$ 2.7	65.8 $\pm$ 5.0
	25	32.5 $\pm$ 3.4	41.8 $\pm$ 4.3
	50	20.8 $\pm$ 10.2	36.3 $\pm$ 3.0
	100	11.7 $\pm$ 1.4	15.7 $\pm$ 2.5

^a The pollen tube length is expressed in μm .

ment of pollen tube length, pollen was sown on this medium in a straight row with a cover glass. After cultivation in a moist chamber for 90 min (for *I. balsamina*, after 30 min) at 25°C , the percentage of germination for 100~150 pollen grains was determined with a microscope, and tube length was measured with an ocular micrometer after 20 hr (for *I. balsamina*, after 15 hr) cultivation. In the case of *P. densiflora*, pollen was sown on the growth medium which consisted of 2% sucrose, 10 ppm H_3BO_3 , 10 ppm MgSO_4 and 10 ppm $\text{Ca}(\text{NO}_3)_2$, the germination percentage and pollen tube length were measured after 50 hr and 168 hr cultivation at 27°C , respectively.

As shown in Table I, pollen germination of *C. japonica* was almost completely inhibited at 100 ppm and pollen tube elongation was also inhibited in proportion to the concentration of benzoic acid at less than 100 ppm. The same results were observed in pollen of other species except *Pinus* pollen. In the case of *P. densiflora*, the inhibition due to benzoic acid was relatively slight, which might be understood by considering that this pollen grains themselves contained an appreciable amount of the inhibitor.³⁾

These results suggested that benzoic acid might be involved in regulating pollen germination or pollen tube elongation at fertilization.

Shibuya *et al.*⁸⁾ isolated (+)-abscisic acid from *P. densiflora* pollen using the lettuce seed germination test as an indicator of biological activity. We found that synthetic abscisic acid inhibited pollen germination of *C. japonica*, *C. sinensis* and *P. densiflora* at 500 ppm. In this communication, another kind of endogenous pollen growth inhibitor was isolated employing the pollen germination test as bioassay.

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mine the germination percentage, pollen was sown on the culture medium containing 10% sucrose, 1.2% agar and 10 ppm H_3BO_3 with a tested compound (pH 5.5). For the measure-

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